Analysis of genetic relationship in 12 species of *Section Strobus* with ISSR markers

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Abstract: Genetic relationship of 12 species of *Section Strobus* was analyzed with ISSR markers. 117 loci were detected with 12 ISSR primers. Percentage of polymorphic bands (PPB) varied from 5.93% to 19.92%. *P. pumila* had the highest levels of genetic differentiation and *P. flexilis* had lowest. Total genetic diversity (H_T) of 12 species in *Section Strobus* was 26.21%, of which intraspecific genetic diversity (H_S) was 7.66%, and interspecific genetic diversity (D_{ST}) was 18.55%, and the genetic variation in interspecies accounted for 70.78% of the total genetic diversity. According to the cluster results of genetic distance, the 12 species were classified into two groups. The first group included *P. griffithii*, *P. armandi*, *P. fenzeliana*, *P. kwangtungensis*, *P. strobus*, *P. monticola* and *P. wangii*. The second group included *P. albicaulis*, *P. pumila*, *P. flexilis*, *P. sibirica* and *P. koraiensis*.

Keywords: *Pinus*; ISSR-PCR; Genetic relationship
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Introduction

Section Strobus of *Subg. Haploxylone*, only over 20 species in the world, mainly distribute in the areas of the East Asia and North America. In China, only 11 species mainly distributed in the areas of Northeast, North China, Central China, Southwest, and Northwest (Wu 1995). Up to now, there is always a doubt about the classification of *Section Strobus*. Traditional classification of *Section Strobus* generally depended on their morphological characters and the difference of isozymes, but as character and character expression were easily affected by environment factors, thus traditional classification system of *Section Strobus* had a certain extent error. Since the early of 1980s, with the development of molecular marker techniques, many marker techniques were used. The genetic composition of plant could be determined by the method of DNA character analysis. The molecular marker techniques of DNA have many advantages, such as exact result and stability. Thus it has been widely used in the analysis of genetic variation of forest population, the construction of gene map with high diversity, and molecular assisted breeding (Zou 2001).

In this study, genetic variations of 12 species for *Section Strobus* were analyzed by ISSR DNA marker technique. The main purpose of the study is to test genetic variation and interspecific relationships between 12 species of *Section Strobus*, present a molecular proof for the classification of *Section Strobus*, and provide a scientific basis for the introduction, breeding and the preservation of genetic resources.

Materials and methods

Most of samples were collected from native areas, and few were collected from introduced areas (Table 1).

Table 1. The sources of *Section Strobus*

Species	Resources	Number of individuals
P. koraiensis	Harbin	14
P. sibirica	Russia	13
P. pumila	Russia	10
	Daxing'an Mountains	15
P. strobus	Jilin	10
P. armandi	Liaoning	12
P. wangii	Yunnan	10
P. fenzeliana	Hainan	10
P. griffithii	Xizang	10
P. kwangtungensis	Guangdong	9
P. monticola	Canada	10
P. albicaulis	Canada	5
P. flexilis	Canada	5

Extraction of total DNA

Total DNA was extracted from embryo or needle of tested samples by the CTAB method. The purity and concentration of total genomic DNA were detected by agarose gel electrophoresis.

ISSR-PCR amplification system and detection of products

PCR amplification of *Section Strobus* was performed according to ISSR-PCR reaction system and reaction procedure (Lu 2003). Total DNA of 12 species of *Section Strobus* were used as template and 12 ISSR primers were used in the PCR reaction (the Sequences of primers were provided by University of British Columbia in Canada) (Table 2). The 20-μl solution of reaction contained 40 ng template, 0.5 pmol·L^{-1} primer, 2 μl 10×PCR buffer (0.2 mmol·L^{-1}), 2 μl dNTP (0.2 mmol·L^{-1}), and 1 U *Taq* polymerase (Promega). The conditions of PCR amplification were: 94 ℃ for 5 min; 94 ℃ for 30 s, 35 cycles; 56 ℃ for 45 s; 72 ℃ for 2 min; and 72 ℃ for 7 min. PCR products were separated in 1.2% agarose gels; meanwhile molecular sizes of the fragments were estimated with a 100 bp DNA ladder (MBI). After electrophoresis, PCR products were detected by UVP Gel Documentation Systems (GDS7600).

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Table 2. Sequence of primers

The list of primers (5'-3')	The sequence of primers	The list of primers (5'-3')	The sequence of primers
834	$(AG)_8YT$	811	$(GA)_8C$
818	$(CA)_8G$	807	$(AG)_8T$
808	$AG)_8C$	835	$(AG)_8YA$
848	$(CT)_8RC$	827	$(AC)_8G$
828	$(TG)_8A$	849	$(GT)_8YA$
836	$(AG)_8YA$	820	$(GT)_8C$

Records of ISSR bands

The DNA bands in the gels were recorded after electrophoresis. Each band, as a molecular marker, represents a pair of complementarily combined loci of primers and its annealing DNA template. According to the corresponding place of DNA ladders in the gels, the sizes of the target fragments can be easily estimated. Number 1 indicated the presence of a single band in certain place and number 0 indicated the absence of a single band. And the 0/1 matrixes were input into computer.

Statistical analysis

Data were processed by software of Popgen32. The Nei diversity index of ISSR-PCR products were computed, the distributions of genetic variation for 12 species of *Section Strobus* were studied, genetic distances of interspecies were calculated, and the dendrogram of the species was reconstructed.

Results and analysis

Percentage of polymorphic bands (PPB)

133 individuals of 12 species in *Section Strobus* were analyzed by ISSR-PCR, 117 loci were detected with 12 primers, whose sizes ranged from 200 bp to 2 500 bp. The PPBs of 12 species present a greatly change, ranging from 9.40% to 33.33% (Table 3), and the corresponding order of the species from big to the small were: *P. pumila* (Russia)>*P. pumila* (Daxing'an Mountains)>*P. albicaulis*>*P. kwangtungensis*>*P. koraiensis*>*P. sibirica*>*P. armandi*>*P. wangii*>*P. fenzelana*>*P. griffithii*>*P. strobus*>*P. monticola*>*P. flexilis*. Of these 12 species analyzed, *P. pumila* has the highest PPB, among which the PPB of *P. pumila* from Russia was up to 33.33%, and that of *P. pumila* from Daxing'an Mountains was 32.48%. Thus, the results showed that the intraspecies genetic variation of *P. pumila* was very high, and this variation widely existed in different sites or populations. The PPB of *P. flexilis* was lowest, only 9.40%.

Analysis of genetic diversity

The genetic diversities of 12 species in *Section Strobus* were estimated by the indexes of Shonnon diversity (*I*) and Nei's gene diversity (*H*). The results showed that Shonnon index varied from 0.0518 to 0.1910 (Table 3). The corresponding variation order of the species from big to small were: *P. pumila* (Russia)>*P. pumila* (Daxing'an Mountains)>*P. albicaulis*>*P. kwangtungensis*>*P. koraiensis*>*P. sibirica*>*P. armandi*>*P. griffithii*>*P. fenzeliana*>*P. strobus*> *P. wangii*>*P. monticola*>*P. flexilis*. Nei index varied from 0.0346 to 0.1306 (Fig. 3), and the order of the species was *P. pumila* (Russia)> *P. pumila* (Daxing'an Mountains)>*P. albicaulis*>*P. kwangtungensis*>*P. koraiensis*>*P. sibirica*>*P. armandi*>*P. wangii*>*P. fenzeliana*>*P. griffithii*> *P. monticola*>*P. strobus*>*P. flexilis*. These results showed that of the 12 species, genetic variation of *P. pumila* was highest, and *P. flexilis* was lowest.

Table 3. Comparison of genetic variation in thirteen pines

Species	Total sites	Number ofpolymorphic loci	PPB/%	Shannon *I**	Nei *H**
P. pumila (Russia)	117	39	33.33 %	0.1910	0.1306
P. pumila (Daxing'an Mountains)	117	38	32.48 %	0.1751	0.1180
P. albicaulis	117	37	31.62 %	0.1706	0.1144
P. kwangtungensis	117	29	24.79 %	0.1465	0.1005
P. koraiensis	117	30	25.64 %	0.1406	0.0948
P. sibirica	117	22	18.80 %	0.1019	0.0687
P. armandi	117	15	12.82 %	0.0818	0.0576
P. wangii	117	16	13.68 %	0.0803	0.0552
P. fenzeliana	117	12	10.26 %	0.0654	0.0458
P. griffithii	117	15	12.82 %	0.0673	0.0449
P. monticola	117	17	14.53 %	0.0691	0.0445
P. strobus	117	16	13.68 %	0.0665	0.0432
P. flexilis	117	11	9.40 %	0.0518	0.0346

Intraspecific and interspecific genetic differentiation

Genetic differentiation of 12 species was estimated by Nei's gene diversity. Total genetic diversity (H_T) of *Section Strobus* tested was 26.21%, of which diversity of intraspecies (H_S) was 7.66%, and genetic diversity of interspecies (D_{ST}) was 18.55%. The differentiation of among species accounted for 70.78% of total genetic diversity (G_{ST}). Thus, the results showed that the level of genetic variation in interspecies was very high.

Analysis of genetic homegeneity and clustering

The interspecific genetic homegeneity of 12 species was in range of 0.6865-0.9801 by the analysis with software Popgen32, of which *P. monticola* and *P. strobus* had the highest genetic homegeneity, namely their genetic differentiations were lowest.

The matrix of genetic distance of 12 species was analyzed with Statistica software and the dendrogram of genetic distance was obtained. The tested species were clustered into two groups at the 0.35 genetical distance. The first group included *P. griffithii*, *P. armandi*, *P. fenzeliana*, *P. kwangtungensis*, *P. strobus*, *P. monticola* and *P. wangii*; the second group included *P. albicaulis*, *P. pumila*, *P. flexilis*, *P. sibirica* and *P. koraiensis*.

Discussion

In this study, genetic variations of 12 species in *Section Strobus* were analyzed using 12 ISSR primers. Genetic variations of

Pinus were estimated by the PPB, Shonnon index and Nei index, and the result showed that the variety of these species were parallel. Of all the species tested, *P. pumila* presented the highest degree of genetic variation, and *P. flexilis* was lowest. Meanwhile, the results also showed that conspecific *Section Strobus* coming from different areas could represent the genetic character of the species (for example, *P. pumila*).

In history, *Section Strobus* was the first classified by Shaw with the character of cone and seed as the criteria. Shaw classified the species of *P. albicaulis*, *P. cembra*, *P. koraiensis*, *P. pumila*, *P. sibirica* into *Subsect. Cembra* in the book "The genus *Pinus*". Since then, these species were always regarded as one group in application (Shaw 1914). From then on, taxonomists argued whether *P. albicaulis* belonged to *Subsect. Cembrae* represented by *P. cembra*, or *Subsect. Strobus* represented by *P. strobus*. Some taxonomists thought that *P. albicaulis* belonged to *Subsect. Strobus*, because it has some common characters in morphological character and ecological structure with some species of *Subsect. Strobus*. According to the relationships reconstructed at DNA level, we thought that *P. albicaulis*, most possibly, belongs to *Subsect. Cembrae*.

The results of genetic cluster showed that *P. pumila* and *P. koraiensis* were classified into the same group, which was consistent with classical classification of *Section Strobus* (Fig.1). However, according to the studies of arrangement mode of cone seminiferous scale in *Section Strobus*, Zhao *et al.* (1998) found that *P. pumila* had different arrangement mode compared with *Subsect. Cembrae*, but it had the same arrangement mode as some represented species of *Subsect. Strobus*. We considered that the reason that *P. pumila* had the similar morphological structure as *Subsect. Strobus* was, most possibly, due to convergent evolution.

In classical classification of *Section Strobus*, *P. armandi* was classified into *Subsect. Strobus*, but some taxonomists classified *P. armandi* into *Subsect. Cembrae* because *P. armandi* on cone arrangement or the type of resin duct was more similar to *Subsect. Cembrae* (Gui *et al.* 1963). On the basis of the results of genetic cluster, we thought that *P. armandi* was likely to belong to *Subsect. Strobus*.

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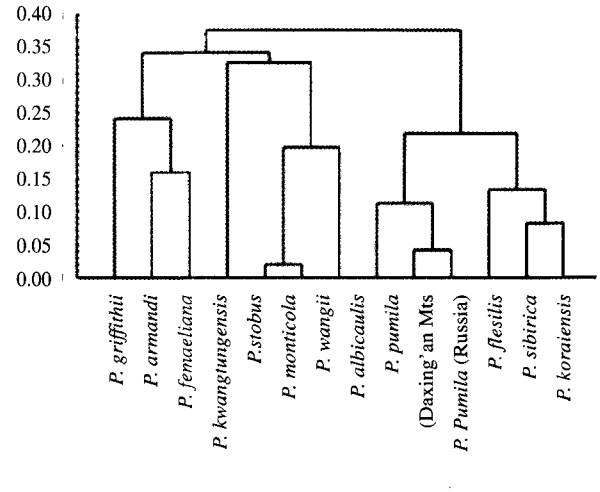

Fig. 1 Dendrogram among *Pinus* species based on the genetic distances generated by Popgen32

References

Gui Yaolin, *et al.* 1963. Comparative dissection observation of *Pinus* needle in China [J]. Acta Botanica Sinica, **11**(1): 44–66. (In Chinese)

Lu Yanfang, Liu Guifeng, Jiang Jing, *et al.* 2003. The ISSR-PCR reaction system's establishment about seed's endosperm of five needles pine [J]. Bulletin of Botanical Research, **23**(4): 429–432.

Shaw, George Russell. 1914. The genus *Pinus* [M]. Arnold Arbor. Publ. **5**: 1–96.

Wu Gang, Feng Zhongwei. 1995. The sociological characteristics and biomass of stone pine forests in China [J]. Acta Ecological Sinica, **15**(3): 260–267

Zhao Guangyi, Liu Wenjie, Zhao Bosheng. 1998. Comparative morphological studies on the three kinds of *Subsect.Cembrae* of *Pinus* from Daxing'an Mountains in China [J]. Journal of Northeast Forestry University, **26**(3): 1–5. (In Chinese)

Zou Yuping, Ge Song, Wang Xiaodong. 2001. Molecular markers in systematic and evolutionary botany [M]. Beijing: Science Press, p167. (in Chinese)